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Alenizi, Dhahi; Ringwood, Tamara; Redhwan, Alya; Bouraha, Bouchra; Wren, Brendan W; Prentice, Michael; McNally, Alan

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All *Yersinia enterocolitica* are pathogenic: Virulence of phylogroup 1 *Y. enterocolitica* in a *Galleria mellonella* infection model.

Dhahi Alenizi[1], Tamara Ringwood[2], Alya Redhwan[1], Bouchra Bouraha[1], Brendan W Wren[3], Michael Prentice[2], Alan McNally[1*]

[1]Nottingham Trent University, Pathogen Research Group, Clifton Lane, Nottingham NG11 8NS

[2]Department of Microbiology, University College Cork, Cork, Ireland

[3]Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT.

[*]Corresponding author: Dr Alan McNally, Nottingham Trent University, Pathogen Research Group, Clifton Lane, Nottingham, NG11 8NS. 0044 1158483324. alan.mcnally@ntu.ac.uk

Abstract

Yersinia enterocolitica is a zoonotic pathogen and a common cause of gastroenteritis in humans. The species is composed of 6 diverse phylogroups, of which phylogroup 1 strains are considered non pathogenic to mammals due to their lack of the major virulence plasmid pYV and their lack of virulence in a mouse infection model. Here we present data examining the pathogenicity of strains of *Y. enterocolitica* across all six phylogroups in a *Galleria mellonellla* model. We show that in this model phylogroup 1 strains exhibit severe pathogenesis with a lethal dose of as low as 10 cfu. We show that this virulence is an active process and that flagella play a major role in the virulence phenotype. Furthermore, we show that the complete lack of virulence in *Galleria* of the mammalian pathogenic phylogroups is not due to carriage of the pYV virulence plasmid. Our data suggest that all *Y. enterocolitica* can be pathogenic, which may be a reflection of the true natural habitat of the species and that we may need to reconsider the eco-evo perspective of this important bacterial species.

Introduction

Yersinia enterocolitica is a member of the Enterobacteriaceae, and a common cause of gastroenteritis in humans (Bottone, 1999). The majority of human infections are associated with consumption of, or contamination from, raw and undercooked pork products (Bottone, 1999; Drummond *et al.*, 2012). Carriage of *Y. enterocolitica* is frequently reported in pig tonsil and intestinal tissues (Martinez *et al.*, 2010; McNally *et al.*, 2004; Milnes *et al.*, 2008) as well as faecal samples from cattle and sheep (McNally *et al.*, 2004; Milnes *et al.*, 2008). Human yersiniosis is generally a sporadic infection (Bottone, 1997; Drummond *et al.*, 2012), however it is the third most common cause of bacterial gastroenteritis in developed countries, behind *Campylobacter* and *Salmonella* (McNally *et al.*, 2004; van Pelt *et al.*, 2003; Rosner *et al.*, 2010). Large outbreaks have also recently been reported with prolonged epidemic curves (Gierczynski *et al.*, 2009).

Y. enterocolitica is classically typed using a series of biochemical utilisation tests which separated the species into six distinct biotypes, and further subdivided by classical serotyping (Bottone, 1999; Wauters *et al.*, 1987). More recently whole genome sequences and phylogenetic studies have shown that biotypes are not phylogenetically robust. This has resulted in a proposed a new nomenclature consisting of phylogroup (PG) 1 (biotype 1A), phylogroup 2 (biotype 1B), phylogroup 3 (serotype O:3), phylogroup 4 (serotype O5;27), phylogroup 5(serotype O:9), and phylogroup 6 (biotype 5)(Hall *et al.*, 2015; Reuter *et al.*, 2014). PG 1 strains are isolated from a wide range of hosts and habitats and are considered to be non-pathogenic due to the lack of pathology in a mouse infection model (Bottone, 1997) and a lack of the major virulence factors found in *Y. enterocolitica* (Bottone, 1999). PG 2 strains are considered high-pathogenic

due to lethality in a mouse infection model, whilst PG 3-6 strains are considered low-pathogenic due to the observed pathology in a mouse infection model (Bottone, 1999). The major genetic difference between PG 1 and PG 2-6 that accounts of the differences in observed pathogenesis are the presence of the virulence plasmid pYV and the adhesion-encoding gene *ail* in PG 2-6 (Reuter *et al.*, 2014).

Despite lacking the key virulence factors involved in mammalian pathogenesis, there is still some debate as to the true pathogenic potential of PG 1 *Y. enterocolitica*. The suitability of the mouse infection model has been questioned as a suitable proxy for human pathogenesis, with different mouse models giving different levels of observed pathology (Schippers *et al.*, 2008). Epidemiological studies have isolated PG 1 strains from humans with gastroenteritis (Mallik & Virdi, 2010; McNally *et al.*, 2004), and experimental studies have shown that PG 1 isolates exhibit the ability to invade cultured epithelial cells (Grant *et al.*, 1999; McNally *et al.*, 2006; Tennant *et al.*, 2003). PG 1 isolates have also been shown to survive inside cultured macrophages for longer time-frames than pathogenic PG 2-5 isolates and to trigger a pro-inflammatory response upon macrophages uptake (McNally *et al.*, 2006). It is known that many PG 1 isolates carry genes that have been proposed to be *Yersinia* virulence factors (Kumar & Virdi, 2012; Singh & Virdi, 2004; Tennant *et al.*, 2005) and population genomic studies have shown that many genes purported to play a role in *Y. enterocolitica* pathogenesis of other PGs are found in PG 1 isolates, and additionally a putative type III secretion system is found exclusively in PG 1 (Reuter *et al.*, 2014). To date the only factor that has been shown to be involved in a virulence associated trait in

PG 1 is the requirement of flagella to survive inside macrophages (McNally *et al.*, 2007b).

Here we present data examining the ability of PG 1 *Y. enterocolitica* to infect the wax-moth insect larvae *Galleria mellonella*, a commonly used alternative infection model for enteropathogens (Gaspar *et al.*, 2009; Senior *et al.*, 2011). Our data shows that PG 1 isolates exhibit severe virulence in infected *Galleria*, with a LD_{50} of just 10 cfu, and that virulence is enhanced at 25°C compared to 37°C. We also show that the severe virulence of PG 1 isolates is in direct contrast to mammalian pathogenic PG 2-5 isolates that exhibit almost no virulent phenotype in *Galleria*. We also show that mutations in potential virulence genes previously identified in PG 1 strains show no effect on the *Galleria* virulence phenotype, but that the loss of flagella function previously shown to be necessary for survival in macrophages also attenuates pathogenesis in *Galleria*. Therefore the term non-pathogenic should not be applied to PG 1 *Y. enterocolitica* given the high levels of entemopathogenesis observed here, and that a more comprehensive understanding of *Y. enterocolitica* ecology is required to fully dissect the lifecycle of this highly diverse bacterial species.

Materials and methods

Bacterial strains and plasmids

A full list of bacterial isolates (Table 1) and plasmids (Table 2) used in this study is provided. *Y. enterocolitica* isolates were collected from human, pig, cattle, and sheep faecal samples (McNally *et al.*, 2004). The isolates investigated in depth in the study represent comprehensively characterised type strains of each phylogroup (McNally *et al.*, 2006) and for which a reference genome sequence has been produced (Reuter *et al.*, 2014). All strains were routinely cultured from

glycerol stocks stored at -80^{o}C using LB agar at 25^{o}C. For all experiments 10 colonies from an agar plate were incubated in 5 ml LB broth at 25^{o}C with shaking at 200rpm for 18 hours. Strains YE8081, YE1203 and YE14902 had pYV minus derivative constructed by 2 x 18 hour serial passages on LB agar at 37^{o}C followed by incubation on CRMOX agar plates at 37^{o}C and selection of large non-pigmented colonies (Farmer *et al.*, 1992). Absence of pYV was confirmed by Kado and Liu gel electrophoresis (Kado & Liu, 1981) and by PCR using primers Yscp1 and Yscp2 to detect the YscP gene present on pYV.

Galleria **infection assay:** *Galleria mellonella* were infected as previously described (Fuchs *et al.*, 2008). *Galleria* larvae were infected with a series of bacterial suspensions containing 10^1, 10^2, 10^3 ,10^4 ,10^5, 10^6 , 10^7 , 10^8 & 10^9 cfu of each *Y. enterocolitica* strain. Each dose was injected sub-cutaneously in 10 µl-aliquots into a group of 10 active *G. mellonella* larvae using a Hamilton syringe. After injection, each group was placed on a separate 90 mm sterile Petri Dish containing a 90mm diameter Whatman filter paper. The injected *Galleria* groups were then incubated in the dark at 25°C or 37°C and monitored for a period of 5 days. Ten larvae were injected with a sterile PBS, and 10 were incubated without any form of injection or treatment. Cessation of movement and changes in larvae cuticle colour were checked to distinguish dead larvae. All experiments were repeated in triplicate independently. The LD_{50} value (the lethal dose required to kill 50% or more larvae after 5 days incubation) was calculated, and statistical significance tests were performed using two-sample T-tests.

For experiments enumerating number of bacteria surviving inside *Galleria* larvae, individual larvae were sacrificed by incision with a scalpel and then ground with a sterile mortar and pestle. The material was then resuspended in

10ml sterile PBS and used for bacterial enumeration using CIN *Yersinia* selective agar.

Mutagenesis of the *cdt* and YGT loci

All mutagenesis studies were performed in the genome sequenced type PG 1 strain YE5303 (McNally *et al.*, 2007; Reuter *et al.*, 2014). A cytolethal distending toxin (CDT) mutant was made by PCR amplifying *cdtB* using primers CDTFor and CDTRev (Table 2) and cloning into pCRTopo2.1 (Invitrogen) to create pAD5. The *cat* gene was PCR amplified from pAM6 using primers CmFor and CmRev (Table 2) and cloned into the *AgeI* site of pAD5. The inactivated *cdtB* gene was then PCR amplified and subcloned into the *SmaI* site of pKNG101. The resulting plasmid was used to transform *E. coli* S17-1 Pir cells, and these were used as donor cells in a filter mating conjugation with YE5303 (McNally *et al.*, 2007). A functional *Yersinia* Genus Type III secretion system (YGT) mutant was constructed by PCR amplifying the apparatus encoding gene *ygtV* (Reuter *et al.*, 2014) using primers ygtvFor and ygtvRev (Table 2) and cloning into pCRTopo2.1 to create pAR1. The *cat* gene was PCR amplified from pAM6 using primers CmFor and CmRev and cloned into an *NheI* site. The inactivated *ygtV* gene was then PCR amplified and subcloned into the *SmaI* site of pKNG101. The resulting plasmid, pAR3, was used to transform *E. coli* S17-1 Pir cells, and these were used as donor cells in a filter mating conjugation with YE5303 (McNally *et al.*, 2007). Complementation of the *cdtB* and *ygtV* mutants was performed by transforming the mutated strains with pAD5 and pAR1 respectively.

Results

Phylogroup 1 *Y. enterocolitica* show high pathogenicity towards *Galleria mellonella*

To determine variation in the pathogenic potential of *Y. enterocolitica* phylogroups to *G. mellonella*, strains YE5303 (PG 1), YE8081 (PG 2), YE1203 (PG 3), YE14902 (PG 4), YE5603 (PG 5) and YE3094/96 (PG 6) were used to inoculate groups of larvae. Bacteria were pre-grown at 25°C and 37°C prior to inoculation, and larvae were also incubated at both temperatures post-infection. The LD_{50} of each strain in each infection condition was calculated and plotted (Fig 1, Fig S1). The data clearly shows that YE8081 of PG 2, the highly pathogenic phylogroup in mouse infection models is the least pathogenic in the *Galleria* assay ($p = 0.001$), and that YE5303 belonging to PG 1, which is considered to be non-pathogenic to mammalian hosts is the most virulent in all conditions tested ($p < 0.00001$) with virulence enhanced at 25°C compared to 37°C, and occurring when larvae were incubated down to as lows as 15°C (data not shown). The mammalian low-pathogenic PG 3-6 strains all showed very low levels of virulence to *Galleria*, with the exception of the PG 4 strain YE14902. To confirm the findings a further 23 strains were tested in the *Galleria* assay (Fig S2) with bacteria pre-grown at 25°C and the infected *Galleria* incubated at 37°C, which show PG 1 strains significantly more virulent in the assay ($p = 0.03$).

Virulence of PG1 *Y. enterocolitica* in *Galleria* is an active process characterised by rapid death

We sought to determine the kinetics of infection by YE5303 in the *Galleria* assay. First we determined the time-to-death for the larvae in all combinations of pre and post inoculation incubation and doses of bacteria (Fig 2). The results show that the vast majority of killing occurs rapidly between 10 and 24 hours after infection, with the exception being doses at or around sub-lethal levels where small numbers of larvae may die after 24-48 hours. We then took lethal doses (1

x 10^9 cfu) and sub lethal doses of YE5303, YE1203, YE14902 and YE8081 (1 x 10^2 cfu, 1x 10^6 cfu, 1 x 10^4cfu, and 1 x 10^7 cfu, respectively) and inoculated *Galleria*. At time intervals we sacrificed 5 x larvae in each group and counted the number of recovered *Yersinia* from each larva (Fig 3). Our data shows that with the mammalian pathogenic strains (YE1203, YE14902, and YE8081) the number of bacteria is unchanged regardless of fate of the larvae. However, in the PG 1 YE5303 the number of bacteria surviving inside the *Galleria* drops dramatically in the 24 hours leading to death, whilst in a sub-lethal dose there is rapid and complete clearance of bacteria. To confirm that the fatal virulence of YE5303 was an active process we prepared serial dilutions of overnight cultures of YE5303 and then heat killed the cells at 60°C for 1 hour (Autenrieth *et al.*, 1994) before injecting larvae. No killing of *Galleria* larvae was observed after injection with any dose of heat killed bacterial cells.

The *Yersinia* virulence plasmid pYV does not have a protective effect on *Galleria* infection

Given the clear difference in pathogenesis in the *Galleria* model between pYV bearing strains and *Y. enterocolitica* PG 1, we sought to determine if pYV was involved in the observed non-pathogenic phenotype of PG 2-6 strains. The pYV plasmid was cured from YE8081, YE1203, and YE14902 by serial culture at 37°C in the absence of calcium ions, and loss of pYV confirmed by PCR and Kado & Liu gel electrophoresis. The plasmid + and plasmid – derivatives were then used to perform larval infections and LD_{50} compared (Fig 4). The data clearly shows that the loss of pYV has no impact on the lack of pathogenesis of pYV bearing strains on *Galleria* larvae. We also checked the stability of pYV during infections by PCR amplification performed on bacteria recovered from dead and surviving larvae

(Fig 4). This shows that pYV was stable in all strains except the PG4 strain YE14902 where 50% (6 of 12 colonies tested by PCR) of tested colonies had lost the plasmid. PG4 strains curiously are also the most virulent of the pYV bearing phylogroups in the *Galleria* assay.

Targeted mutagenesis suggests a role for flagella and intracellular survival in the pathogenesis of PG1 *Y. enterocolitica* to *Galleria* larvae

Comparative analysis of 100 *Y. enterocolitica* genomes spanning the entire species diversity identified two putative virulence factors that are unique or have PG 1 unique alleles (Reuter, *et al.*, 2015). These are the YGT type III secretion system, and the cytolethal distending toxin CDT. A YGT mutant was constructed by insertional inactivation of the *ygtV* apparatus encoding gene, and a CDT mutant by insertional inactivation of the *cdtB* gene. We also utilised a functional flagella mutant made by insertional inactivation of *flgB* previously described by our group (McNally *et al.*, 2007). The mutants and complemented mutants were used to perform *Galleria* infections and LD_{50} calculations (Fig 5). Our data show that mutations in the CDT operon or YGT secretion system have no discernable effect on virulence of YE5303 in *Galleria* larvae. However our previously constructed and characterised flagella mutant has a significant decrease ($p = 0.0014$) in virulence compared to the wild type, with restoration of the phenotype upon complementation with the *flgB* gene on a high copy number plasmid. To test if the lethality may be due to secretion of toxic effectors from the flagella apparatus we tested the lethality of supernatant from overnight cultures of YE5303 and the *flgB* mutant in *Galleria* larvae. Our results showed that supernatant from the wild type YE5303 showed 100% mortality (20/20

larvae) whilst supernatant from the *flgB* mutant showed 10% mortality (2/20 injected larvae).

Discussion

Yersinia enterocolitica is a common causative agent of gastroenteritis in humans and is a zoonotic infection (Valentin-Weigand, P. Heesemann, J. Dersch, 2014). Recent population genomic studies have shown that *Y. enterocolitica* is a highly diverse species composed of six genetically distinct phylogroups (Hall *et al.*, 2015; Reuter *et al.*, 2014; Reuter *et al.*, 2015). The pathogenic potential of each of the phylogroups has been well characterised on the basis of epidemiological studies of human infections (Fredriksson-Ahomaa & Korkeala, 2003; McNally *et al.*, 2004) as well as the use of mouse models of infection (Handley *et al.*, 2004). However there is still discordance between such data sets, with a prime example being the frequent isolation of PG 1 *Y. enterocolitica* from symptomatic humans (Mallik & Virdi, 2010; McNally *et al.*, 2004) despite this lineage lacking the essential pYV virulence plasmid (Reuter *et al.*, 2014) and being completely non-pathogenic in mouse infection models (Schiemann & Devenish, 1982). To further investigate this dichotomy we utilised the *Galleria mellonella* infection model as a novel infection model for representative strain of all *Y. enterocolitica* phylogroups.

Our data shows that the PG 1 *Y. enterocolitica* strains are highly virulent to *G. mellonella* larvae with a lethal dose as low as 10 cfu, with virulence enhanced when the infection is incubated at 25^{o}C compared to 37^{o}C, though there was no difference if the bacteria were pre-incubated at different temperatures prior to infection. Conversely, the so called high-pathogenic PG 2 strains showed virtually no virulence at all using any infection conditions. Additionally, the most

254 frequently encountered human-pathogenic phylogroups showed only trace
255 levels of virulence with infectious doses of 10^7 – 10^9 cfu. These results appear
256 counterintuitive and may suggest that *G. mellonella* is a measure of virulence for
257 insects, but not for human disease, at least for *Y. enterocolitica*.
258 Our data raise more questions on our perceived knowledge of the ecology, life
259 style and evolution of pathogenesis of the *Y. enterocolitica* species. Previous
260 work has shown variation in pathogenesis of the human pathogenic *Yersinia*
261 species in insect models of infection (Fuchs *et al.*, 2008) and that insect toxin
262 genes present in PG 3, 4 and 5 strains of *Y. enterocolitica* only contribute to
263 virulence in insects infected via oral ingestion (Fuchs *et al.*, 2008). Our data
264 clearly shows that PG 1 *Y. enteroclitica* are acutely pathogenic to *Galleria*
265 *mellonella* via direct sub-cutaneous injection whilst the mammalian pathogenic
266 phylogroups are not, and it would now be interesting to test PG 1 strains via oral
267 ingestion by insects as these were not tested in the previous study. The variation
268 in pathogenesis suggests that different phylogroups are exposed to varying
269 predation threats, supporting recent population genomic analysis suggesting
270 that the phylogroups inhabit distinct ecological niches or micro-habitats on the
271 basis of limited gene sharing (Reuter *et al.*, 2015).
272 A key difference between PG 1 *Y. enterocolitica* and the pathogenic phylogroups
273 is the absence of the pYV virulence plasmid, the major virulence determinant in
274 mammalian pathogenic *Yersinia* species (Reuter *et al.*, 2014). The plasmid
275 contains the Ysc type III secretion system which is known to be used by *Yersinia*
276 to disarm macrophages and dendritic cells to allow the bacteria to avoid
277 phagocytosis (Cornelis & Wolf-Watz, 1997). Given that *G. mellonella* are known
278 to contain a functional non-specific immune response it seemed obvious that this

279 may be the reason for the lack of response of the larvae to the mammalian
280 pathogenic phylogroups. However our data shows that loss of pYV had no effect
281 on the virulence of the mammalian pathogenic phylogroups towards the larvae.
282 Indeed our data shows that the virulence of PG 1 *Y. enterocolitica* towards *G.*
283 *mellonella* is an active process that requires live cells. Our finding that heat killed
284 bacterial cells are unable to cause mortality also rule out the possibility that LPS,
285 which is phylogroup specific in *Y. enterocolitica* (Reuter, *et al.*, 2015), is the cause
286 of the severe toxicity of PG 1 strains.
287 Rather our results show a key role for flagella in the pathogenesis of phylogroup
288 1 *Y. enterocolitica* in *G. mellonella*. Previous work by our group showed that fully
289 functioning flagella are required for the ability of a PG 1 strain to survive inside
290 human cultured phagocytic cells for prolonged periods (McNally *et al.*, 2006,
291 2007a). This suggests that the pathogenesis of PG 1 *Y. enterocolitica* to *G.*
292 *mellonella* relies upon the ability of bacteria to survive the interaction with
293 phagocytic cells in the haemoceol (Fuchs *et al.*, 2008). The essential role of
294 flagella in the *G. mellonella* virulence process also explains the increased
295 pathogenesis when infected larvae are incubated at 25^{o}C which is the permissive
296 temperature for flagella gene expression in *Y. enterocolitica* (Kapatral *et al.*,
297 1996). However, the fact that PG 1 strains still show toxicity at 37^{o}C and that the
298 pre-infection incubation temperature has no effect on toxicity suggests that there
299 are other underlying molecular mechanisms of both flagella expression
300 regulation and *G. mellonella* pathogenesis. Indeed there are no apparent
301 differences in flagella structure or amino acid sequence between PG 1 and PG 2-5
302 strains (Reuter *et al.*, 2014; Reuter *et al.*, 2015). This means that the presence of
303 flagella alone is not sufficient to induce toxicity in the larvae, and that PG 1 *Y.*

enterocolitica utilise their flagella differently to other lineages of the species. It may be that PG 1 strains utilise their flagella as a secretion system for lineage specific effector proteins, something which has previously been proposed in the species (Schmiel *et al.*, 2000) and is supported by the data showing that supernatant from wild type PG1 bacteria is lethal to larvae but supernatant from a flagella mutant is not . Alternatively it may be that there is co-ordinated interaction between flagella and another as-yet-unidentified system in the mammalian pathogenic phylogroups that down-regulates the pathogenic phenotype of the flagella. It is known that there is transcriptional regulation interplay between flagella and the Ysc secretion system in *Y. enterocolitica* (Kapatral & Minnich, 1995), and so it is possible that flagella function is differentially regulated in each lineage.

We therefore propose that it is no longer accurate to describe PG 1 *Y. enterocolitica* as non-pathogenic, and that using an insect infection model we show that all phylogroups of the *Y. enterocolitica* are capable of exhibiting high levels of virulence in selected hosts. This emphasises our need to better understand the true ecology of each lineage of this important bacterial species. Furthermore there is now merit to fully investigate the differential functional roles of flagella in each of the *Y. enterocolitica* phylogroups, as well as differences that may exist in their regulatory control.

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472 **Table 1: List of strains used in this study**

Strain Name	Phylogroup	Biological Origin	Source
YE8081	PG1	Human	(Thomson *et al.*, 2006)
YE0902	PG1	Sheep	(Reuter *et al.*, 2014)
YE0903	PG1	Human	(Reuter *et al.*, 2014)
YE3403	PG1	Human	(Reuter *et al.*, 2014)
YE3503	PG1	Pig	(Reuter *et al.*, 2014)
YE5303	PG1	Human	(McNally *et al.*, 2006)
NZ3	PG1	Sheep	(Reuter *et al.*, 2014)
YE11902	PG5	Sheep	(Reuter *et al.*, 2014)
YE21202	PG5	Pig	(McNally *et al.*, 2006)
YE21502	PG5	Pig	(Reuter *et al.*, 2014)
YE21802	PG5	Pig	(Reuter *et al.*, 2014)
YE2403	PG5	Human	(Reuter *et al.*, 2014)
YE5603	PG5	Human	(McNally *et al.*, 2006)
YE5803	PG5	Human	(Reuter *et al.*, 2014)
YE11102	PG4	Sheep	(Reuter *et al.*, 2014)
YE14902	PG4	Sheep	(McNally *et al.*, 2006)
YE15302	PG4	Cattle	(Reuter *et al.*, 2014)
YE22602	PG4	Pig	(Reuter *et al.*, 2014)
YE23102	PG4	Pig	(Reuter *et al.*, 2014)
YE23202	PG4	Pig	(Reuter *et al.*, 2014)
YE01/2012	PG3	Human	Claire Jenkins HPA
YE02/2012	PG3	Human	Claire Jenkins HPA
YE0303	PG3	Human	(Reuter *et al.*, 2014)
YE1203	PG3	Human	(McNally *et al.*, 2006)
YE20102	PG3	Pig	(Reuter *et al.*, 2014)
YE20402	PG3	Human	(Reuter *et al.*, 2014)
YE21302	PG3	Pig	(Reuter *et al.*, 2014)
NZ15	PG3	Pig	(Reuter *et al.*, 2014)
Y1	PG3	Human	Petra Dersch, HZI)
YE3094/96	PG6	Hare	(Reuter *et al.*, 2014)
YE5303-*flgB*Mut		YE5303 with *flgB* gene inactivated	(McNally *et al.*, 2007)
YE5303-*cdtB*Mut		YE5303 with *cdtB* gene inactivated	This study
YE5303-*ygtV*Mut		YE5303 with *ygtV* gene inactivated	This study
E. coli S17-1 Pir			Epicentre UK
E. coli DH5α			Invitrogen UK

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474 **Table 2: List of primers and plasmids used in this study**

Name	Description	Source
pCR2.1-TOPO	TA cloning vector	Invitrogen
pAR1	pCR2.1 with *ygtV* inserted	This study
pAM6	pCR2.1 with *cat* inserted	(McNally *et al.*, 2007a)
pAR2	pAR1 with *cat* inserted	This study
pKNG101	*sacB*/λPir suicide vector	(Kaniga *et al.*, 1991)
pAR3	pKNG101 with Inactivated *ygtV* from pAR2 inserted	This study
pAD5	pCR2.1 with *cdtB* inserted	This study
pAD6	pAD5 with *cat* inserted	This study
pAD9	pKNG101 with inactivated *cdtB* from pAD6 inserted	This study
cdtFor	GGAAATAAATAAATCTGG	Tm 53^{0}C
cdtRev	GGGTGAGTAGAGTACGGT	
ygtFor	GCGCTATATCAGGTAGTTTC	Tm 57^{o}C
ygtRev	CGGGAGAATACCGATGAGAG	
CmFor	ACCGAGCGTAGCGAGTCAGT	Tm 60^{o}C
CmRev	ATTACGCCCCGCCCTGC	
YscP1	ATTAGAACCTGAGTATCAACC	Tm 52^{o}C
YscP2	AACAAATAACTCATCATGTCC	

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Figure 1: The calculated LD_{50} values for infection of *Galleria mellonella* of a cross section of reference *Y. entercolitica* strains representing the diversity of the species. Results show values for strains pre-incubated at both 25°C and 37°C prior to inoculation into larvae. Black bars represent LD_{50} values for infection at 37°C and grey bars represent values for infection at 25°C. Values are the mean for three independent experiments and error bars represent the standard error of the mean.

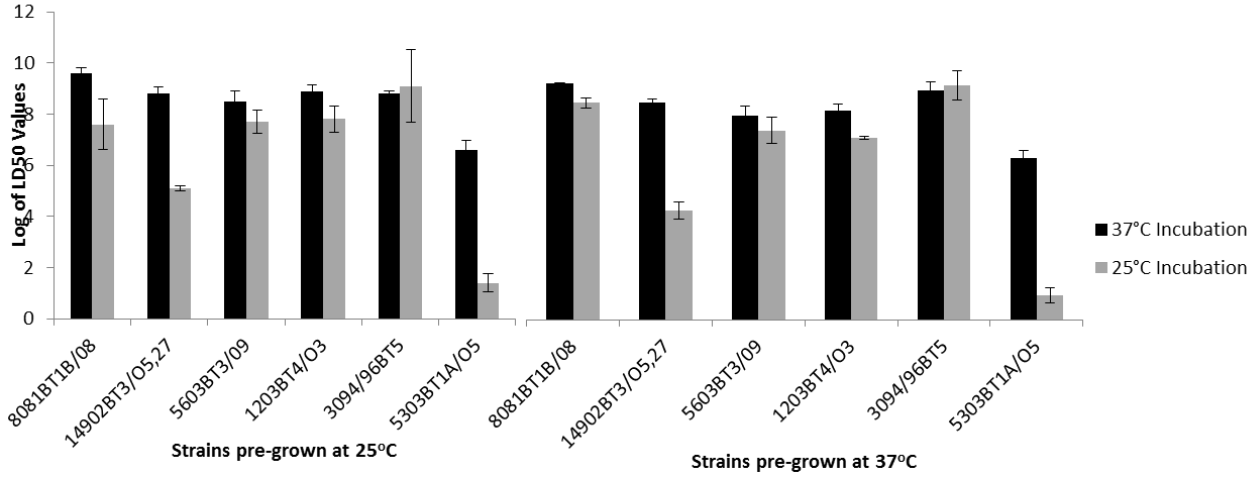
Figure 2: Survival curves for *Galleria mellonella* infected with the phylogroup 1 *Y. enterocolitica* reference strain 5303. Data shown is a representative experiment of three independent replicate experiments.

Figure 3: Infection kinetics graphs showing the numbers of bacteria recovered from infected *Galleria mellonella* larvae infected with reference *Y. enterocolitica* strains at lethal and sub-lethal doses. Data shown are the mean of three independent experiments with the error bars indicating the standard error of the mean.

Figure 4: (A) The effect of the loss of the pYV virulence plasmid on the pathogenesis of reference *Y. enterocolitica* strains to *Galleria mellonella*. Data shown are the mean of three independent experiments with the error bars indicating the standard error of the mean. (B) image showing the stability of pYV in reference strains of *Y. enterocolitica* during *Galleria mellonella* infection, as determined by PCR detection of the *yscP* gene. Lane M contains a 100bp marker; Lanes 1 & 2 show YE8081c 24 hrs and 120 hrs post infection; Lanes 3 & 4 show

12/03 24 hrs and 120 hrs post infection; Lanes 5 and 6 show 212/02 24 hrs & 120 hrs post infection; Lanes 7 & 8 show 149/02 24 hrs & 120 hrs post infection; Lanes 9 & 10 show 56/03 24 hrs and 120 hrs post infection; Lanes 11 & 12 show 3094/96 24 hrs and 120 hrs post-infection. Lane marked –ve is a no template negative control.

Figure 5: Graph showing the effect of mutation and complementation in the CDT operon (cdtB), the YGT type III secretion system (ygtV), and flagella (flgB) on the pathogenesis of the phylogroup 1 *Y. enterocolitica* reference strain 5303. Data shown are the mean of three independent experiments with the error bars indicating the standard error of the mean.


12
10
8
6
4
2
0
Log of LD50 Values
37°C Incubation
25°C Incubation
8081BT1B/08
14902BT3/O5,27
5603BT3/09
1203BT4/O3
3094/96BT5
5303BT1A/O5
8081BT1B/08
14902BT3/O5,27
5603BT3/09
1203BT4/O3
3094/96BT5
5303BT1A/O5
Strains pre-grown at 25°C
Strains pre-grown at 37°C

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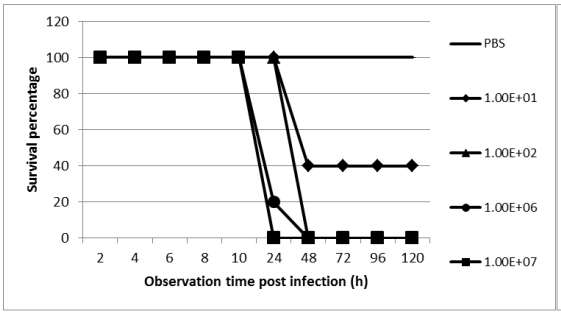
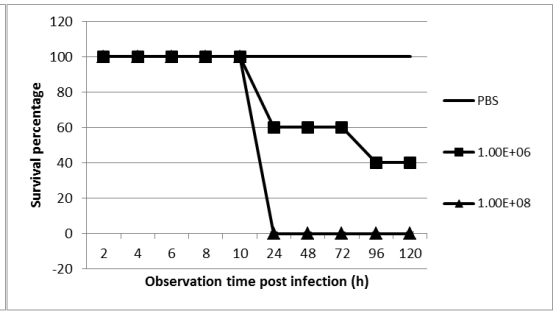
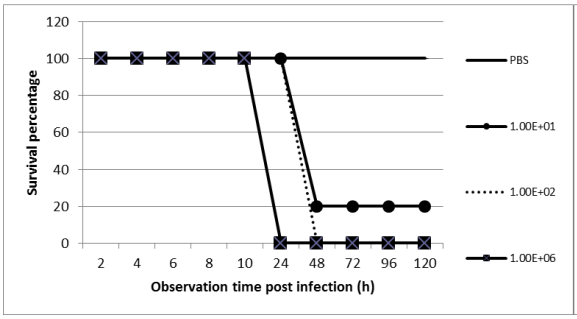
Yersinia 25°C – *Galleria* 25°C



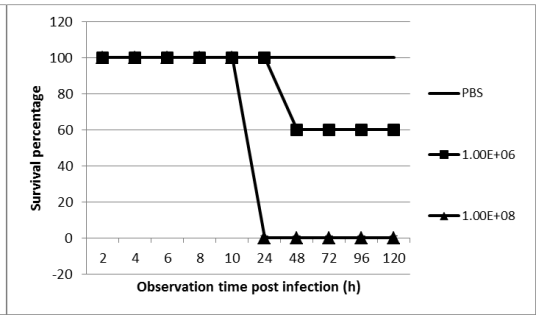
Yersinia 25°C – *Galleria* 37°C



Yersinia 37°C – *Galleria* 25°C



Yersinia 37°C – *Galleria* 37°C



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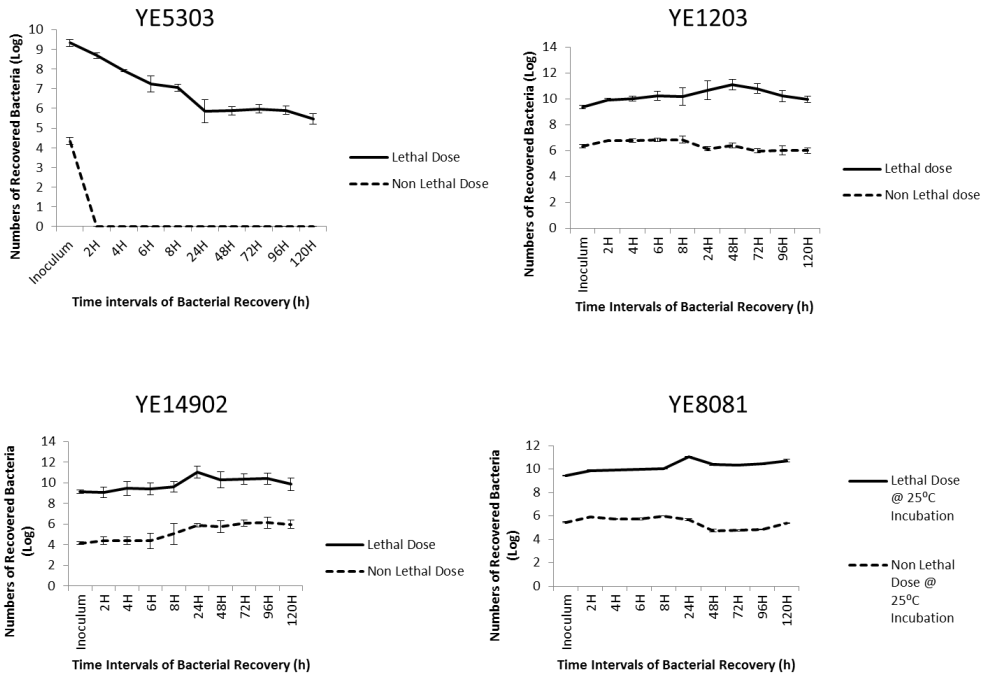
YE5303
Numbers of Recovered Bacteria (Log)
Time Intervals of Bacterial Recovery (h)
Inoculum
2H
4H
6H
8H
24H
48H
72H
96H
120H
Lethal Dose
Non Lethal Dose
YE1203
Numbers of Recovered Bacteria (Log)
Time Intervals of Bacterial Recovery (h)
Lethal dose
Non Lethal dose
YE14902
Numbers of Recovered Bacteria (Log)
Time Intervals of Bacterial Recovery (h)
Lethal Dose
Non Lethal Dose
YE8081
Numbers of Recovered Bacteria (Log)
Time Intervals of Bacterial Recovery (h)
Lethal Dose @ 25°C Incubation
Non Lethal Dose @ 25°C Incubation

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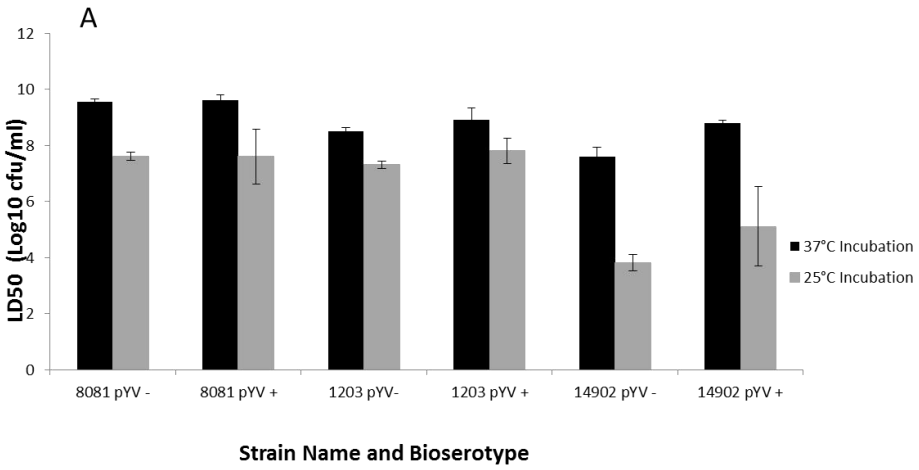
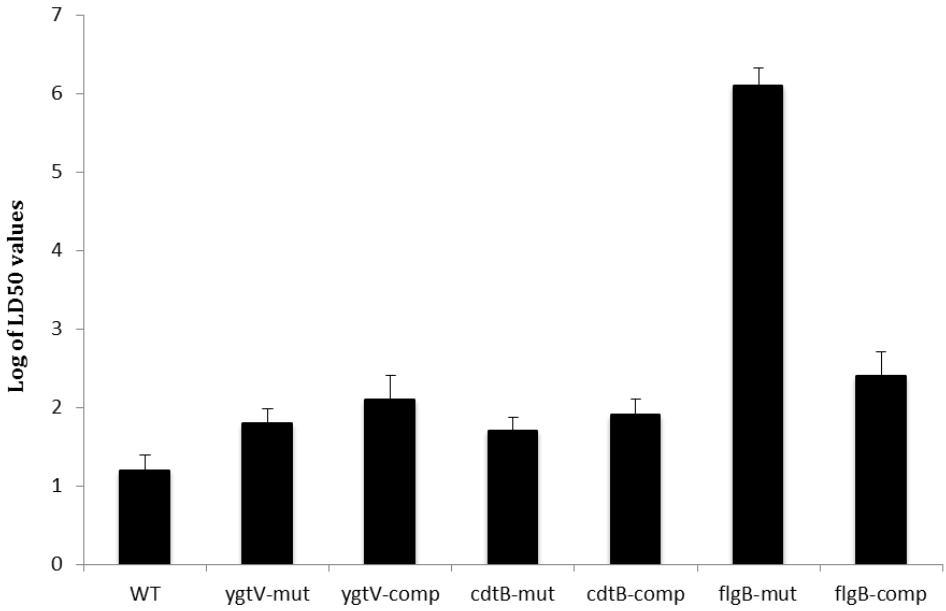

A
12
10
8
6
4
2
0
LD50 (Log10 cfu/ml)
37°C Incubation
25°C Incubation
8081 pYV -
8081 pYV +
1203 pYV-
1203 pYV +
14902 pYV -
14902 pYV +
Strain Name and Bioserotype


B
M
1
2
3
4
5
6
7
8
9
10
11
12
-ve
500bp

524
525


Log of LD50 values
7
6
5
4
3
2
1
0
WT
ygtV-mut
ygtV-comp
cdtB-mut
cdtB-comp
flgB-mut
flgB-comp

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527